Australasian Plant Disease Notes (2023) 18:18
https://doi.org/10.1007/s13314-023-00503-4

Detection of *heterodera mani* in Western Australia

Daniel C. Huston[1] · Mike Hodda[1] · Andrea Hills[2] · Sarah Collins[2]

Received: 29 March 2023 / Accepted: 17 May 2023

Abstract
The ryegrass cyst nematode, *Heterodera mani*, is reported from Western Australia for the first time. Cysts were recovered from soil samples collected on a broadacre cropping property near the town of Esperance. The production area is dominated by cereal/oilseed rotation and a species of annual ryegrass (*Lolium rigidum*) is a common weed issue in these paddocks. Morphometrics of cysts and second stage juveniles (J2s) from the Western Australian population were consistent with data from other reports of this species. Sequences of the mitochondrial cytochrome c oxidase I (COI) gene region were generated and matched those of *H. mani* from previous reports. Sequences of the large subunit ribosomal RNA (28S rRNA) were produced for *H. mani* for the first time. Although interspecific variation is relatively low for this gene in the *Heterodera avenae* species complex, our analyses indicate that 28S gene sequences sufficiently differentiate *H. mani* from other *H. avenae*-group members. *Lolium rigidum* is likely the host for the *H. mani* population discovered, although this requires further confirmation.

Keywords Tylenchoidea · Heteroderidae · Cyst nematode · Ryegrass

The ryegrass cyst nematode *Heterodera mani* was first described parasitising several grasses in Northern Ireland, with perennial ryegrass *Lolium perenne* (Poaceae, Poales) designated as the type-host (Mathews 1971). Other known hosts include the grasses *Alopecurus geniculatus*, *Dactylis glomerata*, *Festuca arundinacea*, *Festuca pratensis*, *Festuca rubra commutate*, *Festuca rubra rubra*, *Glyceria fluitans*, *Lolium multiflorum* and *Vulpia bromoides* (Mathews 1971; Mowat 1974). Mathews (1971) and Mowat (1974) determined that *H. mani* does not reproduce on barley, oats or wheat.

Heterodera mani is a member of the *Heterodera avenae* species-group, differing from the other members in having a J2 stage with robust and deeply concave stylet knobs, as well as combinations of morphological and morphometric features (Subbotin et al. 2010). The species is also reliably distinguishable from other species of *Heterodera* using the cytochrome c oxidase I (COI) and internal transcribed spacer (ITS) gene regions (Subbotin et al. 2003, 2010, 2018; Huston et al. 2022).

In 2022, *H. mani* was reported in Australia for the first time, on a farm in north-western Tasmania (Jain et al. 2022). This nematode had previously been reported throughout Europe, from California in the USA and from South Africa (Subbotin et al. 2010).

During an ongoing study of Australian populations of *Heterodera australis*, a population of *Heterodera mani* was detected on a broadacre cropping property near Esperance in Western Australia. The sample was collected from an area in a paddock commonly cropped with a cereal and oilseed rotation where annual ryegrass *Lolium rigidum* is prevalent as a weed. This finding represents the first record of *H. mani* in Western Australia.

Soil samples were collected from a cereal field on a farm 45 km north-east of Esperance, Western Australia, with a history of infestation with annual ryegrass *L. rigidum*. Soil samples were placed in plastic bags and transported to the Australian National Insect Collection, in Canberra, Australian Capital Territory. Heteroderid cysts were extracted from soil using Cobb's sieving and decanting method (Cobb

✉ Daniel C. Huston
daniel.huston@uqconnect.edu.au

[1] Australian National Insect Collection, National Research Collections Australia, CSIRO, PO Box 1700, Canberra, ACT 2601, Australia

[2] Department of Primary Industries and Regional Development (DPIRD), 3 Baron-Hay Court, South Perth, WA 6151, Australia

Published online: 01 June 2023

1918). Collection sites were initially screened for heteroderid species composition through extraction of DNA from crushed whole cysts. Following preliminary detection of *H. mani* at one collection site, 19 additional cysts were processed for dual molecular and morphological study. Cysts were placed in a drop of water and cut in half using a scalpel blade. Interior contents of cysts were carefully removed using fine dissecting forceps and needles. Eggs and other tissues were transferred directly to tissue lysis buffer and stored frozen until DNA extraction. Vulval cones were mounted on slides in a modified Kaiser's glycerin jelly (see Dioni 2003), and photographs were taken using a ZEISS Axiocam 506 mono camera mounted on a ZEISS Axioscope light microscope. Measurements of vulval plates were taken using ZEISS Blue imaging software (ZEISS, Germany). When available, a subset of second stage juveniles (J2s) recovered from individual cysts were mounted on temporary slides in water and photographed and measured as above. These and additional J2s recovered were then killed in hot water, preserved in 4% formalin, processed to glycerol using the slow method (Hooper 1986) and mounted in glycerol on waxring slides for lodgement in the Australian National Insect Collection. Voucher specimens of vulval cones and J2s are lodged under the accession numbers: 8782–8801.

Genomic DNA was extracted from specimens using a DNeasy Blood and Tissue kit (Qiagen®) following the manufacturer's instructions. Two molecular markers were targeted: the mitochondrial cytochrome c oxidase I (COI) gene region and the large subunit ribosomal RNA (28S rRNA). The COI region was amplified using the forward primer JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) (Bowles et al. 1992) and reverse primer JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) (Derycke et al. 2005). The 28S region was amplified using the forward primer D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse primer D2B (5′-TCG GAA GGA ACC AGC TAC TA′) (Nunn 1992). PCR and clean-up followed Huston et al. (2023) and was the same for both gene regions. PCR products were sent to the Biomolecular Resource Facility, Australian National University, Canberra, for Sanger sequencing and resultant reads were assembled and edited using Geneious Prime® v2022.1.1 (Biomatters). Newly generated sequences were aligned with those of other members of the *Heterodera avenae* species group using MUSCLE (Edgar 2004) as implemented in MEGA 11 (Tamura et al. 2021). Sequences of *Heterodera glycines* and *Heterodera schachtii* were used as outgroups. Differences between taxa were examined in MEGA 11 with Neighbour-joining analyses (Saitou and Nei 1987) using the number of differences method with inclusion of transitions and transversions and complete deletion of gaps and missing data; nodal support was estimated with 500 bootstrap replications. Phylogenetic trees and photographic images were edited and annotated in Adobe Illustrator CS6.

Sequence data was successfully generated for 15 cysts collected. Initial BLAST (Altschul et al. 1990) results of COI sequences indicated that all 15 cysts represented *H. mani*. The 15 newly generated COI sequences and seven 28S sequences of *H. mani* lacked intraspecific variation, thus only a single sequence of each gene was submitted to GenBank (COI: OQ918095; 28S: OQ918098). The COI sequences of *H. mani* from Western Australia did not differ (i.e., 0% divergence) from those from Tasmania, Australia or those from Germany or the USA. 28S sequence data was not available for *H. mani* prior to the present study. The newly generated 28S sequences of *H. mani* differed from those of *Heterodera aucklandica* by 3 base positions (bp), from *Heterodera avenae* by 2 bp, from *Heterodera filipjevi* by 6–7 bp, from *Heterodera hordecalis* by 2–11 bp, from *Heterodera latipons* by 14–15 bp, and from *Heterodera pratensis* by 2 bp. Neighbour-joining analysis of the COI dataset placed the Western Australian sequence in a clade with those of *H. mani* from other regions with high support (Fig. 1). Analysis of the 28S dataset (Fig. 2) resolved *H. mani* as sister to a clade comprising sequences of *H. avenae* and a single sequence of *H. aucklandica*, *H. hordecalis* and *H. pratensis*.

Morphological specimens were consistent with the concept of *H. mani*. Cysts were bifenestrate with conspicuous bullae and an underbridge was not conspicuous (Fig. 3). Morphometrics of the vulval plate (Table 1) were within the range reported for this species (see Subbotin et al. 2010). Morphometric data obtained from J2s (Table 1) were also consistent with previous reports for this species (Subbotin et al. 2010; Jain et al. 2022). Stylet knobs of J2s were well-developed and exhibited the deeply concave anterior faces (Fig. 3) characteristic of this life stage in this species (Subbotin et al. 2010).

Huston et al. (2022) showed that 28S gene sequences were generally not sufficient to distinguish between several members of the *H. avenae* species group and the 28S sequences of *H. mani* generated here differ by as little as 2 bp from some *H. avenae* group members. However, the present results indicate that 28S sequences can reliably delineate *H. mani* from related species. This is similar to the situation regarding sequences of the internal transcribed spacer region (ITS; comprising ITS1-5.8S-ITS2). Although ITS sequences of *H. mani* differ by only 2 bp from some sequences of *H. avenae*, these sequences of *H. mani* were found to consistently form a monophyletic clade in phylogenetic analyses (Huston et al. 2022).

The present finding of *H. mani* in Western Australia, coupled with the recent first detection of this species in Tasmania (Jain et al. 2022) suggests *H. mani* is likely widespread

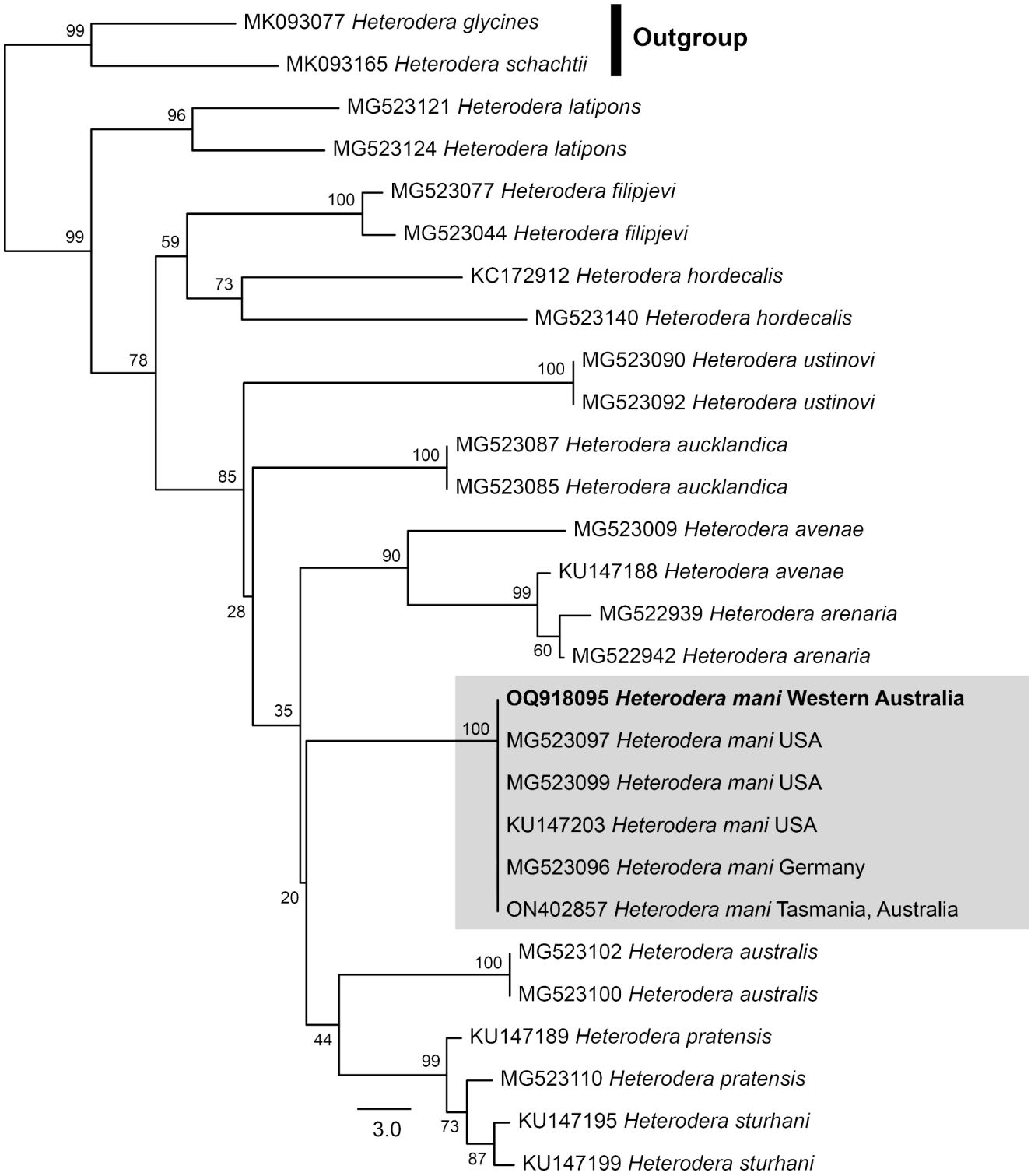

Fig. 1 Phylogram from the neighbour-joining analysis of the COI mtDNA dataset. Sequence of *Heterodera mani* generated in this study shown in bold. Bootstrap support values are shown at the nodes. The scale bar indicates the number of base differences

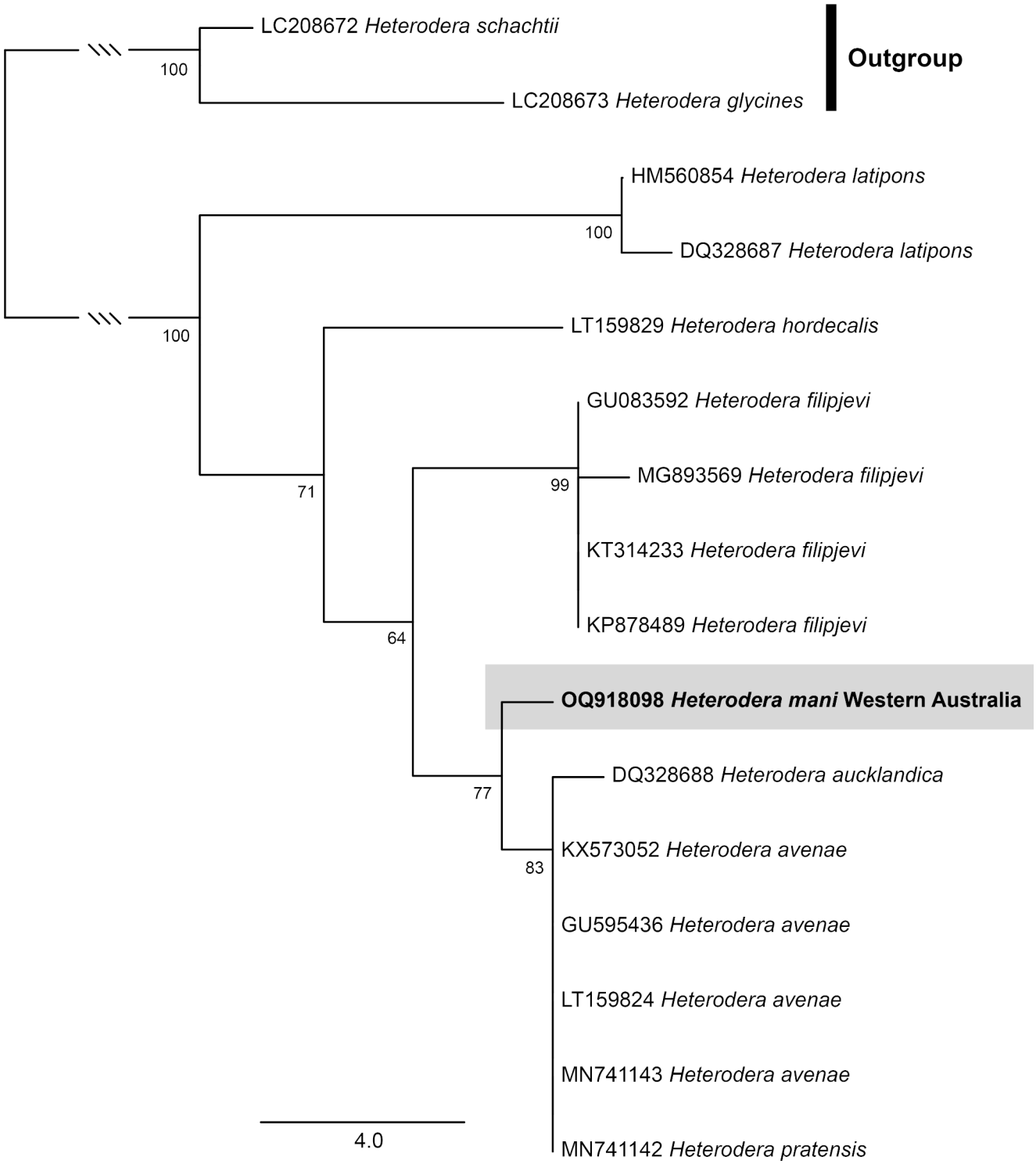

Fig. 2 Phylogram from the neighbour-joining analysis of the 28S rDNA dataset. Sequence of *Heterodera mani* generated in this study shown in bold. Bootstrap support values are shown at the nodes. The scale bar indicates the number of base differences

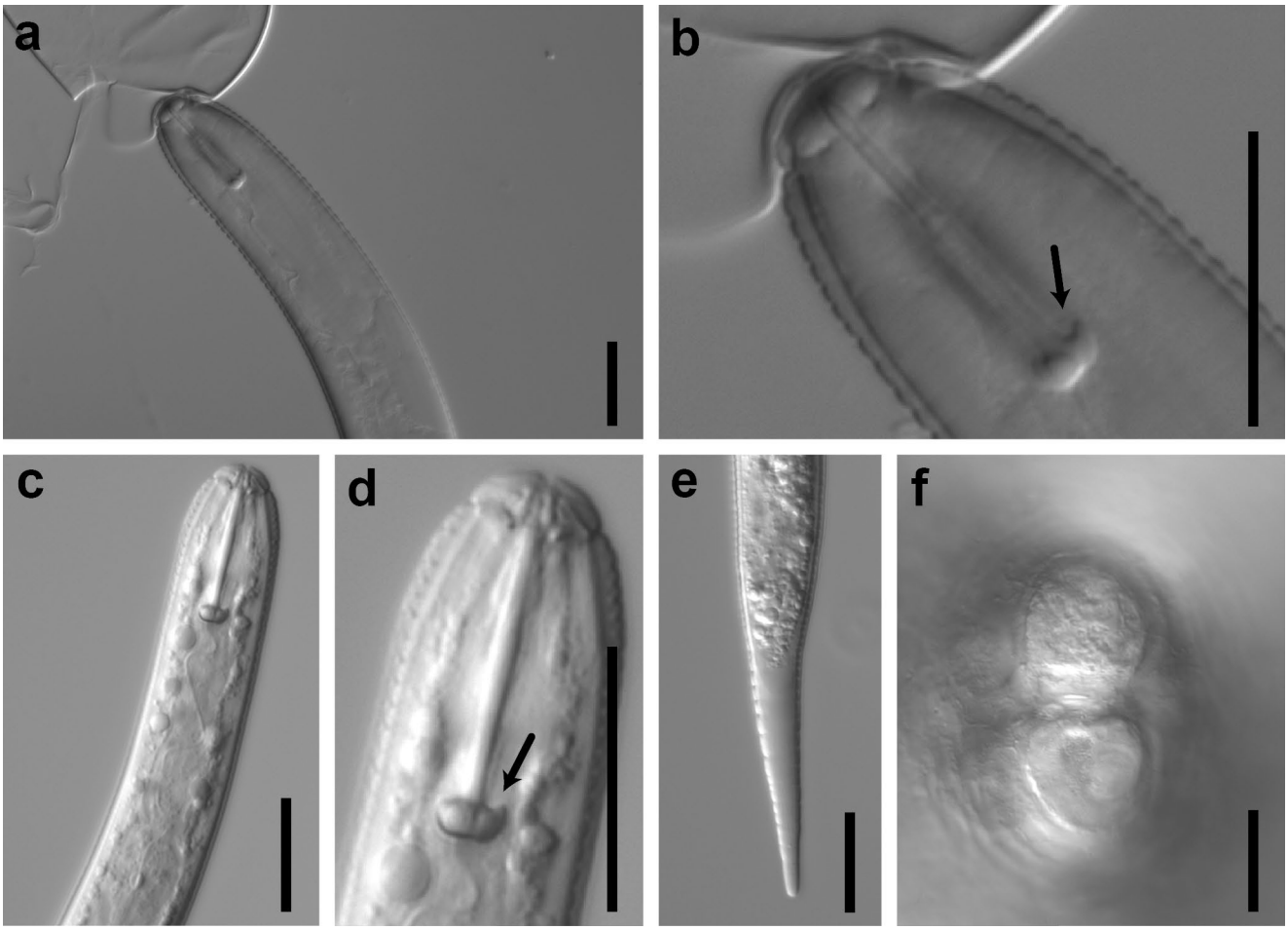

Fig. 3 *Heterodera mani* second stage juveniles (**a-e**) and vulval plate (**f**). (**b**) and (**d**) are enlargements of (**a**) and (**c**), respectively; (**e**) hyaline region of tail. Arrows indicating the deeply concave stylet knobs characteristic of *H. mani*. Scale bars = 20 µm

throughout Australia. Grass species of the genus *Lolium* are thought to be hosts for *H. australis* (McLeod 1992) but considering the subtle morphological differences between this species and *H. mani* (see Subbotin et al. 2010), it is possible that at least some reports of cyst nematodes from *L. rigidum* were *H. mani* rather than *H. australis*. *Heterodera mani* has been reported from *Lolium multiflorum* and *Lolium perenne* (Mathews 1971; Mowat 1974), but we are not aware of any previous records from *L. rigidum*. Because *Heterodera mani* is not known to parasitise cereals or oilseeds, it seems likely that *L. rigidum* is the host for the *H. mani* population discovered. However, confirmation of *L. rigidum* as a host will require observation of white females on the roots of this plant. *Heterodera mani* has probably been present in Australia for many years but had not been recognised as distinct from *H. australis* until recent molecular work.

Annual ryegrass is the most common ryegrass weed present in Australian dryland cropping systems (Jones et al. 2000). A cultivar of this species, cv. Wimmera, is sometimes sown in dryland areas for grazing purposes where other ryegrass species do not persist due to the Mediterranean climate (hot, dry summers) or where greater tolerance of soil salinity is required. Ryegrasses are typically inhabited by endophytic fungi which facilitate production of alkaloids that can have negative impacts on grazing insects and other animals (Popay et al. 2021; Karpyn Esqueda et al. 2017). Although endophytes have been reported to provide some protection against a variety of insect pests, including root-feeding species (Karpyn Esqueda et al. 2017; Popay et al. 2021), data on deterrence of plant parasitic nematodes has largely been inconclusive (Cook et al. 1991; Bell et al. 2009; Stewart et al. 1993; Eerens et al. 1998; Panaccione et al. 2006). Evaluating the potential for endophytic fungal-conferred protection against *H. mani* in ryegrasses may be warranted. The damage cyst nematodes cause cereal crops suggests investigations into the capacity of endophytic fungi to confer protection against these pests could be invaluable.

Table 1 Morphometrics of vulval region and second stage juveniles of *Heterodera mani* from Western Australia. Measurements are presented in the form: mean ± standard deviation (range). Abbreviations: Morphometric indices (a-c, b', c') follow Hooper (1986)

Feature	Measurement
Vulval area (n = 11)	
Fenestral length	52.1 ± 2.6 (47.0–54.4)
Fenestral width	23.1 ± 2.3 (19.3–26.7)
Vulval slit length	9.5 ± 1.0 (7.4–10.5)
Vulval bridge width	9.4 ± 1.6 (7.0–11.9)
Vulva to anus	48.8 ± 0.4 (48.5–49.0)
J2s (n = 12)	
Length	558.8 ± 15.2 (536.3–582.1)
Body diameter at mid-body	23.3 ± 0.86 (22.0–24.8)
Tail length	69.1 ± 2.9 (65.3–76.0)
Diameter of body at anus	16.6 ± 1.13 (14.4–18.6)
Hyaline region length	41.9 ± 3.9 (36.2–51.0)
Labial region height	5.1 ± 0.28 (4.8–5.6)
Labial region diameter	10.5 ± 0.54 (9.6–11.4)
Stylet	26.7 ± 0.53 (25.9–27.4)
Dorsal gland opening to stylet base	5.8 ± 1.3 (3.5–7.8)
Dorsal gland opening to anterior end	34.5 ± 1.5 (31.9–37.2)
Anterior end to median bulb valve	79.4 ± 3.7 (72.8–84.9)
Anterior end to excretory pore	112.8 ± 5.3 (103.8–123.3)
Oesophagus length to oesophageal intestinal valve	127.9 ± 7.0 (117.0–141.7)
Oesophagus length to end of glands	223.5 ± 30.4 (193.7–264.7)
a	23.9 ± 1.1 (22.2–25.5)
b	4.4 ± 0.29 (3.8–4.8)
b'	2.5 ± 0.37 (2.0–2.8)
c	8.1 ± 0.29 (7.7–8.7)
c'	33.6 ± 2.3 (31.3–39.5)
Hyaline region / stylet length	1.6 ± 0.16 (1.4–1.9)
Length / anterior end to median bulb valve	7.1 ± 0.41 (6.4–7.0)

Acknowledgements We thank the growers and Joel Kidd (DPIRD) who kindly provided samples which were used as part of this study. This project is supported by Grains Research and Development Corporation, through funding from the Australian Government Department of Agriculture, Fisheries & Forestry, as part of its Rural R&D for Profit program and along with Cotton Research and Development Corporation, Hort Innovation Australia, Wine Australia, Sugar Research Australia and Forest and Wood Products Australia.

Funding Open access funding provided by CSIRO Library Services.

Declarations

Conflict of interest The authors have no conflict of interest to declare that are relevant to this article.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bell NL, Rohan TC, James SM, Aalders LT, Burch G, Sarathchandra SU, Gerard E, O'Callaghan M (2009) An investigation on non-target impacts of ryegrass endophytes on nematodes and soil microorganisms. P N Z Grassl As 71:139–144. https://doi.org/10.33584/jnzg.2009.71.2742

Bowles J, Blair D, McManus DP (1992) Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol Biochem Parasitol 54:165–173. https://doi.org/10.1016/0166-6851(92)90109-W

Cobb NA (1918) Estimating the nema population of soil with special references to the sugarbeet and root-gall nemas, *Heterodera schachtii* Schmidt and *Heterodera radicicola* (Greef) Muller, and with a description of *Tylencholaimus aequalis* n. sp. Agricultural Technol Circular 1

Cook R, Lewis GC, Mizen KA (1991) Effects on plant-parasitic nematodes of infection of perennial ryegrass, *Lolium perenne*, by the endophytic fungus, *Acremonium lolii*. Crop Prot 10:403–407. https://doi.org/10.1016/S0261-2194(06)80032-3

Derycke S, Remerie T, Vierstraete A, Backeljau T, Vanfleteren J, Vincx M, Moens T (2005) Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. Mar Ecol Prog Ser 300:91–103. https://doi.org/10.3354/meps300091

Dioni W (2003) Safe microscopical reagents for amateurs. Mounting microscopic subjects. Part 4 -Glycerine jellies. Part 5 - mountant summary. Micscape Magazine. Microscopy UK

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. https://doi.org/10.1093/nar/gkh340

Eerens JPJ, Visker MHPW, Lucas RJ, Easton HS, White JGH (1998) Influence of the ryegrass endophyte (*Neotyphodium lolii*) in a cool moist environment IV. Plant parasitic nematodes. New Z J Agric Res 41:209–217. https://doi.org/10.1080/00288233.1998.9513304

Hooper DJ (1986) Handling, fixing, staining and mounting nematodes. In: Southey JF (ed) Laboratory methods for work with plant and soil nematodes. vol 6th edition. Her Majesty's Stationery Office, London, UK, pp 59–80

Huston DC, Khudhir M, Hodda M (2022) Reliability and utility of standard gene sequence barcodes for the identification and differentiation of cyst nematodes of the genus *Heterodera*. J Nematol 54:1–24. https://doi.org/10.2478/jofnem-2022-0024

Huston DC, Khudhir M, Hodda M (2023) Phylogenetic position of *Ptychaphelenchus eucalypticola* Hodda, 2009 within the Aphelenchoidoidea Skarbilovich, 1947 (Siddiqi, 1980) inferred from partial 18S and 28S rDNA gene sequences. Nematol 25:59–76. https://doi.org/10.1163/15685411-bja10206

Jain A, Wainer J, Huston DC, Hodda M, Dinh Q, Mann R, Rodoni B, Edwards J (2022) First report of ryegrass cyst nematode, *Heterodera mani*, in Tasmania, Australia. https://doi.org/10.1094/PDIS-05-22-1129-PDN. Plant Dis

Jones R, Alemseged Y, Medd R, Vere D (2000) The distribution, density and economic impact of weeds in the australian annual winter cropping system. Technical Series, vol 4. CRC for Weed Management Systems

Karpyn Esqueda M, Yen AL, Rochfort S, Guthridge KM, Powell KS, Edwards J, Spangenberg GC (2017) A review of perennial ryegrass endophytes and their potential use in the management of african black beetle in perennial grazing systems in Australia. Front Plant Sci 8:1–21. https://doi.org/10.3389/fpls.2017.00003

Mathews HJP (1971) Two new species of cyst nematode, Heterodera mani n. sp. and H. iri n. sp., from Northern Ireland. Nematologica 17:553–565

McLeod RW (1992) On the origin and spread of *Heterodera avenae* in Australia. Nematol Mediterr 20:59–61

Mowat DJ (1974) The host range and pathogenicity of some nematodes occurring in grassland in Northern Ireland. Rec Agric Res 22:51–58

Nunn GB (1992) Nematode molecular evolution: an investigation of evolutionary patterns among nematodes based upon DNA sequences. Ph. D. Thesis, University of Nottingham, Nottingham, UK

Panaccione D, Kotcon J, Schardl C, Johnson R, Morton J (2006) Ergot alkaloids are not essential for endophytic fungus-associated population suppression of the lesion nematode, *Pratylenchus scribneri*, on perennial ryegrass. Nematol 8:583–590. https://doi.org/10.1163/156854106778614074

Popay AJ, Hume DE, Mace WJ, Faville MJ, Finch SC, Cave V (2021) A root aphid *Aploneura lentisci* is affected by *Epichloë* endophyte strain and impacts perennial ryegrass growth in the field. Crop Pasture Sci 72:155–164. https://doi.org/10.1071/CP20299

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Stewart TM, Mercep CF, Grante JL (1993) Development of *Meloidogyne naasi* on endophyte-infected and endophyte-free perennial ryegrass. Australas Plant Pathol 22:40–41. https://doi.org/10.1071/APP9930040

Subbotin S, Sturhan D, Rumpenhorst HJ, Moens M (2003) Molecular and morphological characterisation of the *Heterodera avenae* species complex (Tylenchida: Heteroderidae). Nematol 5:515–538. https://doi.org/10.1163/156854103322683247

Subbotin SA, Mundo-Ocampo M, Baldwin JG (2010) Systematics of Cyst Nematodes (Nematoda: Heteroderinae). Part B. Brill

Subbotin SA, Toumi F, Elekçioğlu IH, Waeyenberge L, Tanha Maafi Z (2018) DNA barcoding, phylogeny and phylogeography of the cyst nematode species of the Avenae group from the genus *Heterodera* (Tylenchida: Heteroderidae). Nematol 20:671–702. https://doi.org/10.1163/15685411-00003170

Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120